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Functional interactions between voltage-gated Ca^{2+} channels and Rab3-interacting molecules (RIMs): New insights into stimulus–secretion coupling

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ABSTRACT

Stimulus–secretion coupling is a complex set of intracellular reactions initiated by an external stimulus that result in the release of hormones and neurotransmitters. Under physiological conditions this signaling process takes a few milliseconds, and to minimize delays cells have developed a formidable integrated network, in which the relevant molecules are tightly packed on the nanometer scale. Active zones, the sites of release, are composed of several different proteins including voltage-gated Ca^{2+} (Ca_V) channels. It is well acknowledged that hormone and neurotransmitter release is initiated by the activation of these channels located close to docked vesicles, though the mechanisms that enrich channels at release sites are largely unknown. Interestingly, Rab3 binding proteins (RIMs), a diverse multidomain family of proteins that operate as effectors of the small G protein Rab3 involved in secretory vesicle trafficking, have recently identified as binding partners of Ca_V channels, placing both proteins in the center of an interaction network in the molecular anatomy of the active zones that influence different aspects of secretion. Here, we review recent evidences providing support for the notion that RIMs directly bind to the pore-forming and auxiliary β subunits of Ca_V channels and with RIM-binding protein, another interactor of the channels. Through these interactions, RIMs regulate the biophysical properties of the channels and their anchoring relative to active zones, significantly influencing hormone and neurotransmitter release.

1. Introduction

There is hardly any cellular function that is not influenced directly or indirectly by transient changes in the intracellular concentration of Ca^{2+}. Stimulus–secretion coupling, a process that connects the receipt of a stimulus with the release of molecules from membrane bound vesicles by exocytosis, illustrates nicely the role of Ca^{2+} as a triggering and controlling event in cell behavior. A classical example of this process is the link between membrane depolarization at the presynaptic terminal and the release of neurotransmitter into the synaptic cleft. However, stimulus–secretion coupling is an essential process occurring not only in neurons but in all secretory cells including, neuroendocrine, endocrine, and exocrine cells.

Secretion of hormones and neurotransmitters involves an elaborate molecular dialogue between voltage-gated Ca^{2+} (Ca_V) channels and the exocytotic machinery. The temporal precision of exocytosis requires a tight spatial coupling between docked vesicles and plasma membrane Ca^{2+} channels. The opening and closing of these channels by depolarizing stimuli, such as action potentials, allows Ca^{2+} ions to enter cells down a steep electrochemical gradient, producing the transient intracellular Ca^{2+} signals that triggers exocytosis. As a consequence, regulation of Ca^{2+} channel activity through activation of second messenger cascades or by protein–protein interactions modulates hormone release and synaptic transmission.

Experimental evidence suggests that Ca^{2+} channels are indeed physically coupled to various proteins in the vesicle release machinery such as syntaxin 1, SNAP-25, and synaptotagmin [1] which enables a short diffusional distance between the channels and the Ca^{2+} sensor for vesicle fusion. However, the list of proteins and molecular interactions that enable an enrichment of Ca^{2+} channels at the active zones is far from complete. Indeed, the presynaptic active zone of synapses consists of a dense accumulation of cytomatrix proteins. Among them, the scaffolding Rab3-interacting molecules (RIMs), has received increasing attention because they appear to play a crucial role in Ca_V channel localization and regulation. Hence, the purpose of the present paper is to review recent advances in our understanding of the interaction between Ca_V channels and RIMs and its possible physiological significance.

2. Rab3-interacting molecules (RIMs)

RIM proteins were originally identified as putative effectors for the synaptic vesicle protein Rab3 [2]. Though a single gene has been detected in invertebrates, experimental data show that in vertebrates

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RIMs are encoded by a complex gene family of four members (RIM1–4). Of these, the RIM2 gene includes three independent promoters that specify the three isoforms of the RIM2 protein, whereas the other three genes appear to have only a single promoter that directs transcription of either RIM1 or RIM3 and RIM4 [3,4]. However, the multiplicity of RIMs in vertebrates is also related to extensive alternative splicing. Three canonical sites of alternative splicing referred to as sites A, B and C has been identified in the sequence analyses of the RIM genes. In addition to any intrinsic activity of the alternatively spliced sequences, alternative splicing may alter the way Rab3 and RIM-binding proteins (RBPs) bind to distinct RIM domains [3,4].

All known RIM proteins share a common structural and functional domain architecture (Fig. 1A), with a C2B module at their C termini [3] which serves as an interaction site with multiple binding partners. RIM1α and RIM2α (hereafter referred to as RIM1 and RIM2) are highly homologous proteins expressed in distinct patterns in the brain and other tissues (Table 1), and are the only members of the protein family that bind Rab3 [5,6]. They are formed by an N-terminal Zn^{2+} finger, a central PDZ domain and two "degenerated" C2-domains which fail to bind Ca^{2+}. These functional domains confer RIM1 and RIM2 a central role as scaffold proteins able to interact with a variety of proteins involved in exocytosis (Fig. 1A) including SNAP25, synaptotagmin, 14-3-3, ELSK/CAST, RBPs and Ca_V channels [7-11]. These molecular interactions regulate vesicle fusion, and therefore contribute to determine the process of hormone and neurotransmitter release triggered by depolarization-induced Ca^{2+} influx. Furthermore, at least one of these two RIM variants is required for effective synaptic transmission [5].

The RIM protein family also includes RIM1β and RIM2β members, which differ from the α-RIMs by the lack of the zinc finger, and RIM2γ–4γ proteins comprising only the C-terminal C2 domain and a flanking N-terminal region [4]. These proteins have been also shown to regulate exocytosis [3]. Excellent reviews have been written on RIM proteins with different emphases, mostly on their role in synaptic plasticity and neurotransmitter release, which are the most studied molecular functions of these proteins [12-14].

3. Voltage-gated Ca^{2+} (Ca_V) channels

Ca_V channels are transmembrane proteins that mediate Ca^{2+} ions to enter to the cell from the extracellular space in response to membrane depolarization, coupling the electrical signals in the cell surface to intracellular processes such as muscle contraction, hormone secretion or neurotransmitter release, among many others [15-18]. According to their electrophysiological properties, Ca_V channels have been classified into low voltage-activated (LVA or T-type) and high voltage-activated (HVA) channels, a class that includes the L-, N-, P/Q- and R-types, which can be distinguished using pharmacological approaches [16,17,19]. Ca^{2+} channels are also named using the chemical symbol of the permeating ion (Ca) with the principal physiological regulator (voltage) indicated as a subscript (Ca_V). The numerical identifier corresponds to the Ca_V channel transmembrane pore-forming α_1-subunit gene subfamily (1 to 3 at present) and the order of discovery of the α_1-subunit within that subfamily. Hence, the Ca_V1 subfamily ($Ca_V1.1$ to $Ca_V1.4$) includes channels containing α_{1S}, α_{1C}, α_{1D}, and α_{1F} which mediate L-type currents. The Ca_V2 family ($Ca_V2.1$ to $Ca_V2.3$)

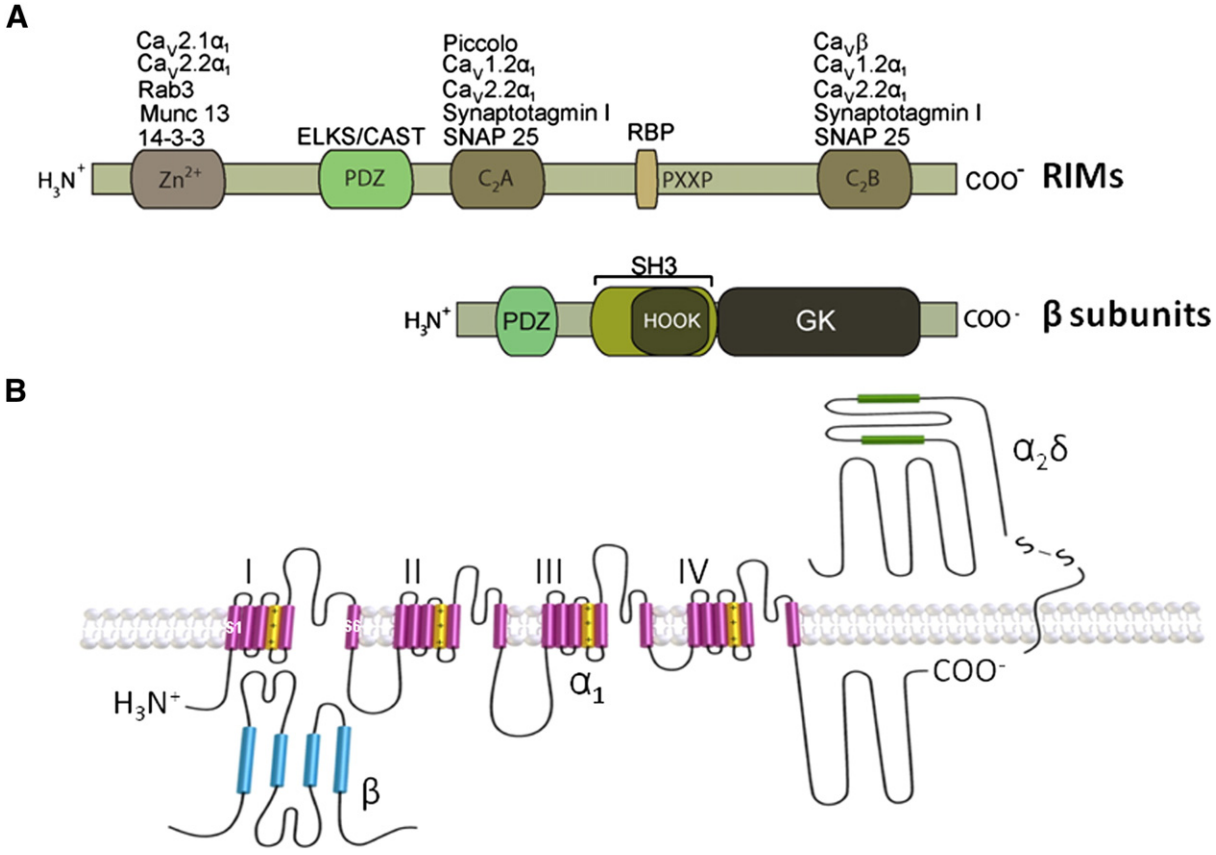

Fig. 1. Molecular structure of RIM proteins, $Ca_V\beta$ subunits, and the HVA Ca_V channel complex. A) RIM 1/2α is formed by one Zn^{2+}-finger-like domain (Zn^{2+}), one PDZ domain (PDZ), two C2 domains (C2A and C2B) and a proline-rich region (PXXP). RIMs 1/2β lack the Zn^{2+}-finger-like domain and RIMs 2γ–4γ have comprised only the C2 domains. $Ca_V\beta$ subunits are formed by three conserved domains: PDZ, Scr homology 3 (SH3) and guanylate kinase (GK). The β-interaction domain (BID) is one of the regions involved in the interaction of the protein with the $Ca_V\alpha_1$ pore-forming subunit. Both protein structures are depicted in N- to C-terminal direction. B) HVA Ca_V channels are oligomeric proteins of a pore-forming α_1 subunit, and auxiliary subunits β and $\alpha_2\delta$. The α_1 subunit is formed by four homologous membrane-spanning repeated domains (I–IV), each with six transmembrane-helixes (S1–S6) and a pore-forming reentrant loop between S5 and S6. The voltage sensor is located in S4 and is indicated in yellow.

Table 1
Summary of RIM isoforms expressed in different cell types and tissues.

Tissue/cell line	Specie	Isoform	Reference
Brain	Rat	RIM1α/RIM2α/RIM2β/RIM2γ/RIM3γ/RIM4γ	[2-5]
	Mouse	RIM1α/RIM2α/RIM2β/RIM3γ/RIM4γ	[5,43,48]
Hippocampus	Rat	RIM1α	[9]
	Mouse	RIM1α/RIM1β/RIM2α/RIM2β/RIM2γ	[6,57,61,77-80]
Cerebellum	Rat	RIM1α/RIM2α/RIM3γ/RIM4γ	[11,43]
	Mouse	RIM1α	[79,81-83]
Calyx of Held	Rat	RIM1α/RIM2α	[84]
	Mouse	RIM1α/RIM2α/RIM1β/RIM2β	[60]
Inner hair cells (immature)	Mouse	RIM2α	[48]
Organ of Corti (immature)	Mouse	RIM1α/RIM2α/RIM2β/RIM3γ	[48]
Eye	Rat	RIM1α/RIM2α/RIM3γ/RIM4γ	[43]
Photoreceptor cells	Rat	RIM1α	[2]
	Mouse	RIM1α/RIM2α	[48,49]
	Human	RIM1α	[58]
Ciliary ganglion calyx	Chicken	RIM1α	[45,85]
Dorsal root ganglion	Rat	RIM1α/RIM2α/RIM3γ	[43]
Spinal cord	Rat	RIM1α	[2]
	Mouse	RIM1α/RIM2α	[5]
Adrenal chromaffin cells	Bovine	RIM1α/RIM2α	[8]
PC12	Rat	RIM1α/RIM2α/RIM3γ/RIM4γ	[11,43]
Pancreas	Rat	RIM2α/RIM4γ	[43]
β Pancreatic cells	Rat	RIM1α	[86]
	Mouse	RIM2α	[56,87]
RIN-m5f	Rat	RIM1α	[47]
INS-1	Rat	RIM2α	[7,51]
MIN-6	Mouse	RIM2α	[50,56]
HIT-T15	Hamster	RIM1α	[86]

includes channels containing α_{1A}, α_{1B}, and α_{1E}, which originate the N-, P/Q- and R-type currents, respectively. Last, the T-type channels are named $Ca_V3.1$ through $Ca_V3.3$, corresponding to α_{1G}, α_{1H}, and α_{1I} [16,17,19].

The expression of the $Ca_V1.1$ channels is restricted to skeletal muscle where they play key roles in excitation–contraction coupling and regulation of gene transcription [19,20]. Hypokalemic periodic paralysis and malignant hyperthermia sensitivity are human diseases associated to mutations in the gene encoding the $Ca_V1.1\alpha_1$ subunit [20-22]. The $Ca_V1.2$ isoform is preferentially expressed in cardiac muscle where it is responsible of excitation–contraction coupling, enzyme activity regulation and gene transcription. The $Ca_V1.3$ isoform displays differential expression patterns in many cell types and tissues including neurons, smooth muscle and inner ear cells as well as in pancreatic islets [20,22,23]. The Timothy and Brugada syndromes have been shown to be associated with mutations in the CACNA1C gene which encodes the $Ca_V1.2\alpha_1$ subunit. Both diseases are characterized by abnormal electrocardiogram findings and an increased risk of sudden cardiac death. The $Ca_V1.4$ channels are expressed in the retina and their function is related to visual transduction. Different mutations in the gene encoding the $Ca_V1.4\alpha_1$ subunit have been associated to an X-linked form of congenital stationary night blindness [20-22].

The $Ca_V2.1$ and $Ca_V2.2$ channels are primarily localized in nerve terminals and are responsible for neurotransmitter release [19,20]. Mutations in the gene coding the $Ca_V2.1\alpha_1$ subunit cause several neurologic disorders including familial hemiplegic migraine type 1, episodic ataxia type 2 and spinocerebellar ataxia type 6 [21,24]. Likewise, $Ca_V2.3$ channels are also expressed in neurons and are involved in repetitive firing, dendritic Ca^{2+} transients and neurotransmission.

Last, Ca_V3 (LVA; T-type) channels are mainly expressed in neurons and cardiac myocytes and its role has been related to pacemaking and repetitive firing. Interestingly, a mutation in one of the three T-type channel isoforms ($Ca_V3.2$) has been associated with childhood absence epilepsy [19,20].

In addition to the $Ca_V\alpha_1$ subunit, Ca_V1 and Ca_V2 channels arise from the multimerization of other proteins including a mostly extracellular $Ca_V\alpha_2\delta$ subunit and a cytoplasmic $Ca_V\beta$ subunit (Fig. 1B). The $Ca_V\alpha_1$ subunit is the principal component of the channels and is responsible for their unique biophysical and pharmacological properties. However, proper trafficking and functioning of the channels require the auxiliary subunits.

The $Ca_V\alpha_2\delta$ auxiliary subunit is a glycosylated protein encoded by a single gene that is proteolytically processed to generate two peptides (α_2 and δ) linked by a single disulfide bridge [25]. The principal effect of this subunit is to increase the functional expression of the channels [26-29], as a consequence of increased trafficking [28]. Structurally the mature $Ca_V\alpha_2\delta$ subunit has been viewed as a type I transmembrane (TM) spanning protein (Fig. 1B), and from the functional point of view there is now compelling evidence for a major involvement of this protein in targeting the Ca^{2+} channel complex to lipid rafts [30-33]. Although a recent study has challenged this model and offered a new mechanism for Ca_V channel raft localization by suggesting the $Ca_V\alpha_2\delta$ subunit associates with the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor in the δ peptide [34], raft localization of $Ca_V\alpha_2\delta$ seems to be preserved even after replacement of the reported GPI anchoring motif with the TM domain of a functionally inert protein PIN-G. Conversely, the GPI-anchoring motif is not sufficient to target PIN-G to lipid rafts [33]. These data are most consistent with a model where $Ca_V\alpha_2\delta$ retains its type I TM topology and its targeting to lipid rafts is governed by sequences upstream of the putative GPI anchor that promote protein–protein rather than lipid–lipid interactions [33].

The $Ca_V\beta$ auxiliary subunits also regulate the functional expression and the biophysical properties of the Ca_V1 and Ca_V2 channels [17,35-37]. Two mechanisms have been proposed for this, an enhancement of expression and a direct effect on gating. With respect to the effect on expression, the $Ca_V\beta$ subunits have been suggested to enhance channel trafficking to the membrane by masking an endoplasmic reticulum retention signal in the $Ca_V\alpha_1$ subunit [35,38] as well as by protecting the channel complex from proteasomal degradation [39,40]. The $Ca_V\beta$ subunits have also been found to hyperpolarize the voltage dependence of activation and increase the channel open probability, which increase current through individual channels and therefore result in augmented macroscopic current density [36,41,42]. The $Ca_V\beta$ auxiliary subunits have a modular structure

consisting of five distinct regions (Fig. 1A). The first, third, and fifth regions are variable in length and amino acid sequence, whereas the second and fourth regions are highly conserved and are homologous to the Src homology 3 (SH3) and guanylate kinase (GK) domains, respectively [36]. These domains are interaction modules engaged in protein–protein interactions.

4. Interactions between Ca_V channels and the RIM family members

Although it is well established that Ca^{2+} entering cells through Ca_V channels in the vicinity of docked vesicles is primarily responsible for initiating hormone release and synaptic transmission, the mechanisms that maintain Ca_V channels at release sites are virtually unknown. Initial studies by Mori and colleagues (2007) have identified a direct interaction between the $Ca_V\beta$ auxiliary subunit and RIM1 by performing yeast two-hybrid screening, pull down and co-immunoprecipitation assays [11]. Using different constructs with several structural motifs of $Ca_V\beta_4$ as bait, they determined that the SH3 and GK domains (Fig. 1A) were necessary for the interaction with RIM1. They also found an interaction with the $Ca_V\beta_2$ subunit, indicating that the association of RIM1 with the channel complex was not dependent on the $Ca_V\beta$ isoform [11]. More recently, the same group extended these studies and showed that members of the four subfamilies of $Ca_V\beta$ subunits bind RIM1, RIM2, RIM3γ and RIM4γ, using GST fusion RIM proteins incubated with cell lysates obtained from transfected HEK-293 cells. Though the affinity of $Ca_V\beta$ for RIMγs seemed to be lower than that for RIMαs [43], its interaction has also important functional consequences as we shall discuss later.

4.1. RIMs may interact with Ca_V channels via its β auxiliary subunits

In their seminal paper, Kiyonaka and co-workers (2007) identified the RIM1 C-terminus as a major $Ca_V\beta$ binding domain by using *in vitro* pull down assays. In the brain, the association of RIM1 with native Ca_V channels (of the $Ca_V2.1$ class) was determined by co-immunoprecipitation assays. Interestingly this association was disrupted when the channels were incubated with a dominant negative construct of $Ca_V\beta_4$ (BADN), which is capable of binding RIM1 but not the $Ca_V\alpha_1$ subunit, providing evidence for a physical interaction between the channel and the synaptic protein in a more physiological context. Likewise, in cultured hippocampal neurons, RIM1 and $Ca_V\beta_{4b}$ both accumulated near presynaptic termini in parallel with $Ca_V2.1$ clustering, suggesting that the RIM1–$Ca_V\beta$ interaction may regulate the localization of channels at the presynaptic membrane [11].

It is worth mentioning also that the Stanley group has argued against the idea of the physical association between RIMs and Ca_V channels based mainly on co-immunoprecipitation experiments using different antibodies against RIM and the $Ca_V2.2$ channel [44-46]. Though the reason for this discrepancy is presently unknown, according to these authors the differing results may be reconciled, in a model where Ca_V channels are interconnected by a scaffold and together form the backbone of the active zone release apparatus; a separate scaffold projects from the channel to the docked secretory vesicles. The model accommodates attachment of new vesicles and their release and, hence, the link between the channels and the vesicles could be cycling between high- and low-affinity states [44-46]. In this scenario, RIMs could be components of the complex, tethering the docking site/vesicle to the channel and responsible for the high- to low-affinity switch.

Recently, our group found that the coupling between RIM1 and the $Ca_V\beta$ auxiliary subunits is also operational in L-type channels ($Ca_V1.2$ and $Ca_V1.3$). By using co-immunoprecipitation experiments, a RIM1–Ca_V channel complex formed by direct interaction of the $Ca_V\beta_2$ and $Ca_V\beta_3$ subunits was identified in a heterologous system and also in RIN-m5F cells, an insulin-secreting cell line [47]. Consistent with this, Striessnig and his colleagues demonstrated that the presence of the auxiliary subunit of the Ca_V channels is necessary for the interaction of RIM1 with the $Ca_V1.3$ channel complex heterologously expressed in tsA-201 cells, and showed also that RIM proteins are expressed in cochlear inner hair cell, where they substantially modify the biophysical properties of the channels [48]. The possible interaction of L-type channels and RIMs is also supported by the observation that the $Ca_V1.3\alpha_1$ subunit is localized at the active zone of retinal photoreceptor ribbon synapses forming tight clusters with different proteins including RIM2 [49].

4.2. RIMs may also bind directly to the Ca_V channel ion-conducting α_1 subunit

A direct interaction between the pore-forming $Ca_V\alpha_1$ subunit and members of the RIM protein family has been also documented [7,50,51]. The first evidence of this interaction was obtained using GST fusion proteins containing the C2 domains of RIMs immobilized on glutathione-agarose beads and incubated with *in vitro* translated proteins representing the cytoplasmic loop connecting domain II and III of $Ca_V1.2\alpha_1$ and $Ca_V2.2\alpha_1$ subunits [7]. These experiments showed that two binding sequences in the C2 domains, KRRT and KKKT, appear to be important for the interaction of RIMs and the II-III loop of the $Ca_V2.2\alpha_1$ subunit.

Likewise, the KKKT sequence in the C2 domain is allegedly responsible for binding the exocytosis-regulatory protein synaptotagmin I, through the synaptic protein interaction (synprint) site of neuronal Ca_V channels (N- and P/Q-type) located in the cytoplasmic loop connecting domains II and III of the pore-forming subunit [52,53]. Encouraged by these findings, Shibasaki and colleagues examined whether RIM2 and another scaffolding protein of the active zone called Piccolo, both of which have C2 domains, also interact with the $Ca_V\alpha_1$ subunit. Their results indicated that the C2 domains of RIM2 and Piccolo bind directly to the cytoplasmic loop of the $Ca_V1.2\alpha_1$ subunit of the L-type channels [50]. It is worth mentioning that these two proteins have been proposed as downstream effectors of EPAC2 (exchange protein activated directly by cyclic AMP type 2) a cAMP-regulated guanine nucleotide exchange factor (GEF) that mediate protein kinase A (PKA)-independent signal transduction properties of the second messenger cAMP [54,55]. Piccolo dimerizes with RIM2 in a Ca^{2+}-dependent manner [56], and both are implicated in potentiation of glucose-stimulated insulin secretion via EPAC2 in pancreatic β-cells. These studies also suggest that Ca_V channels and the secretory granules are targeted to the same region in the pancreatic β cell and that the molecular organization is critical in regulated exocytosis within a zone of voltage-dependent Ca^{2+} entry [50].

In addition, Jacobo and her colleagues using peptides heterologously expressed in insulinoma INS-1 cells confirmed the interaction of the $Ca_V1.2\alpha_1$/II-III loop with RIM2 and Piccolo, and also demonstrated that the II-III loops of $Ca_V1.2\alpha_1$ and $Ca_V1.3\alpha_1$ target their respective channels to membrane microdomains (lipid rafts) possibly through physical interactions with other lipid raft-resident proteins including RIM2. This association appears to be critical for efficient potentiation of glucose-dependent insulin secretion by the hormone glucagon-like peptide-1 in pancreatic β-cells, given that RIM2, acting as an anchor that positions the $Ca_V1.2$ channels within a lipid raft domain, may serve as an assembly point for effectors of cAMP signaling via EPAC2 [51].

Recent studies by the Südhof group have addressed questions about how the N- and P/Q-type Ca_V channels are selectively recruited to presynaptic active zones. By using C-terminal sequences of these channels as baits and distinct fragments of RIM1 as preys they localized the interaction domain to the PDZ region of RIM1. Then, to test whether RIMs act to localize Ca_V channel to active zones, the authors generated conditional double knockout (KO) mice of all RIM isoforms that contain PDZ-domains and using electrophysiology and Ca^{2+}-imaging, as well as quantitative immunostaining of the channels, they

showed that the RIM PDZ-domains are required for localizing the channels to the release sites. They also found that the RIM proline-rich sequences which interact with RBPs also play a key role in tethering the channels to the presynaptic active zones [57]. A possible role for RBPs in synaptic transmission by creating a functional link between the synaptic-vesicle tethering apparatus and the Ca_V channels had been previously suggested by Hibino and colleagues (2002).

Thus, it has been proposed that RIMs tether Ca_V channels to active zones via distinct parallel interactions, i) by directly binding of the $Ca_V\alpha_1$ subunit with the RIM PDZ-domains (interaction that is specific for N- and P/Q-type channels), ii) by indirectly interacting through RBPs, and iii) by association via the $Ca_V\beta$ auxiliary subunit. The latter two types of interaction are shared by different types of channels [10,11,43,57]. As we shall discuss in the next section, the formation of RIM–Ca_V channel complexes has relevant physiological implications.

5. Physiological relevance of the interaction between Ca_V channels and RIMs

The kinetic analysis of the ion channel macroscopic currents has revealed some interesting aspects related to the functional significance of the RIM–Ca_V channel coupling. The most prominent effects of RIMs on Ca_V channel currents occur in the inactivation parameters (Fig. 2). RIM1 drastically slows current inactivation through neuronal Ca_V2 and L-type $Ca_V1.2$ and $Ca_V1.3$ channels [11,47]. Similar effects have been reported for RIM2 on $Ca_V2.1$ and $Ca_V1.3$ channel currents [43,48]. Interestingly, $Ca_V2.1$ current inactivation may be also markedly decelerated by RIM3γ and RIM4γ [43] suggesting that changing the time course of inactivation is a common feature of RIM regulation on Ca_V channel currents.

As mentioned earlier, the studies of Kiyonaka and colleagues (2007) revealed a physical association between RIM and the $Ca_V\beta$ auxiliary subunits that results in a marked suppression of voltage-dependent inactivation of different neuronal Ca_V channels. Consistent with this, docking of secretory vesicles and acetylcholine (ACh) release is significantly enhanced by transfection of RIM1 constructs in neuroendocrine PC12 cells. Conversely, transfection of a dominant negative β construct that disrupts the RIM1–$Ca_V\beta$ association, accelerated inactivation of native channels, suppressed vesicle docking as well as ACh release in PC12 cells, and produced a significant inhibition of glutamate release in cultured cerebellar neurons.

It should be mentioned that RIMγ knockdown attenuates glutamate release in a less pronounced manner than RIMα in cerebellar neurons and its over-expression enhances exocytosis in PC12 cells [3,43]. Interestingly, RIMγs also block localization of the vesicles near the cell membrane [43], confirming that changes in channel function may be a general feature of RIM-mediated regulation, whereas anchoring the vesicles to the channels may depend on the type RIM involved in the interaction [43].

Taking advantage of genetic analyses showing an association between the gene encoding RIM1 and the autosomal dominant cone-rod dystrophy CORD7 [58], the Mori group tested whether the RIM1 mutation associated to CORD7 affected Ca_V regulation by RIM1 [59]. Their data raised the interesting possibility that CORD7 phenotypes including retinal deficits and enhanced cognition are at least partly due to altered regulation of presynaptic Ca_V channels. Hence, a nucleotide (G/A) substitution, that replaces Arg-655 with His in the middle C2A domain, which binds to Ca_V channels, was introduced in the RIM1 cDNA to yield a construct which carried a mutation corresponding to the human CORD7 mutation. Functional analysis showed that in P/Q-type $Ca_V2.1$ channels, the CORD7 mutation enhanced the RIM1-mediated suppression of inactivation and augmented current density, likely leading to enhanced neurotransmitter release and synaptic transmission. In contrast, for L-type $Ca_V1.4$ channels, the CORD7 mutation abolished the RIM1-mediated hyperpolarization of current activation, likely resulting in impaired synaptic transmission at ribbon synapses of the visual system [59].

Heterologous expression of L-type $Ca_V1.3$ channels together with RIM constructs have revealed also that RIMs slow both Ca^{2+}-dependent inactivation (CDI) and voltage-dependent inactivation (VDI) through binding to the $Ca_V\beta$ auxiliary subunits [48]. By slowing inactivation over a large voltage range and by substantially increasing the non-inactivating component of the macroscopic current, RIM-associated channel complexes may carry larger $Ca_V1.3$ window currents during

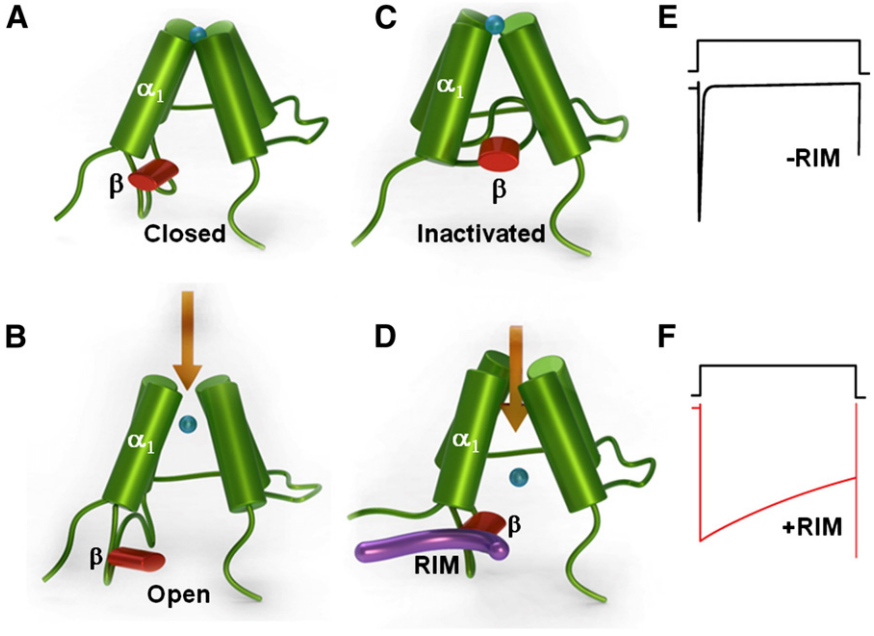

Fig. 2. A hypothetical scheme of the regulation of Ca_V channel inactivation by RIM proteins. Although the crystallographic structure of Ca_V channels is not available, the model is compatible with many experimentally derived data [87]. A) At rest, the channels are closed. B) When an action potential arrives, the channels open allowing the flow of Ca^{2+} ions down their electrochemical gradient. C) During sustained membrane depolarization the channels undergo a conformational change in which the inactivation gate occludes the ion-conducting pore. D) The presence of the RIM proteins results in noticeable changes in channel function, in particular a significant slowdown in inactivation kinetics, as illustrated in the scheme showing the time course of hypothetical macroscopic current traces through Ca_V channels in absence (E) or presence (F) of RIMs.

prolonged depolarization. Interestingly, neurotransmitter release and spontaneous action potentials during cochlear inner hair cell (IHC) development depend on the activity of $Ca_V1.3$ channels which voltage- and Ca^{2+}-dependent inactivation kinetics are slower than in other tissues. It has been reported that RIM2 mRNA is expressed in immature cochlear IHCs and the protein co-localizes with $Ca_V1.3$ channels in the same presynaptic compartment of IHCs [48]. Therefore, association with RIMs may represent a possible molecular mechanism to inhibit CDI and VDI of Cav1.3 channels in IHCs which may be important for the specific signaling functions of these channels during different developmental stages with immature IHCs generating spontaneous, regenerative Ca^{2+} action potentials important for normal development.

Likewise, Gandini and her colleagues (2011) studied the effect of RIM1 on recombinant L-type $Ca_V1.3$ as well as of the $Ca_V1.2$ channels expressed in HEK-293 cells and their functional impact on insulin secretion. Their results show that RIM1 does not alter Ba^{2+} current density through these channels but drastically slows down their inactivation kinetics. As a consequence, the amount of charge mobilized is increased during depolarization, which means that a larger amount of ions passed through the channels when RIM1 is present. Interestingly, the effect of RIM1 on channel inactivation was prevented when $Ca_V\beta$ was absent, confirming that the interaction between the channels and RIM1 may occur via the auxiliary subunit. The relevance of this association was assessed by electrophysiological recordings and insulin secretion measurements in the insulinoma RIN-m5f cells after RIM1 gene silencing. Knock down of endogenous RIM1 increased inactivation of whole cell native currents and decreased insulin release triggered by Ca^{2+} influx in response to K^+-induced membrane depolarization [47].

Using a combined experimental strategy based on protein/protein interaction studies, generation of conditional RIM1/2 knockout mice and electrophysiology, recent studies have shed new light on the physiology of RIMs–Ca_V channel interaction [57,60,61]. Kaeser and colleagues (2011) proposed that RIMs have two alternative forms of interaction with Ca_V channels: a direct one via their PDZ-domains that is specific for N- and P/Q-type channels, and an indirect (non-specific) interaction via RBPs. Using rescue experiments in RIM-deficient hippocampal neurons, these authors found that the RIM PDZ-domain is required to reverse the impairment in presynaptic Ca^{2+} influx in RIM-deficient cells and for localizing the channels to the presynaptic boutons [57]. Similar to that in hippocampal synapses, several lines of evidence suggested that genetic elimination of RIMs interfered with the coupling between Ca_V channels and transmitter release at the calyx of Held, a glutamatergic synapse in the auditory brainstem accessible to quantitative analysis of transmitter release [60].

These studies also show that at both the hippocampal and the calyx of Held synapses, the size of the releasable pool of vesicles is reduced in the RIM1/2 double knockout mouse [57,60,61], corroborating that RIMs are central organizers of active zones. Interestingly, the Zn^{2+} finger domain of RIMs seems to be necessary and sufficient for the vesicle priming function, and given this site interacts with Munc13, it was suggested that the RIMs effects on priming are mediated by Munc13 specifically by preventing its homodimerization [61].

It is well established that G protein coupled receptors (GPCRs) orchestrate precise regulation neurotransmitter and hormone release through inhibition of Ca_V2 channels [62,63]. A unifying property of this GPCR-mediated inhibition of Ca_V is its sensitivity to pertussis toxin, thus implicating $G\alpha_i$ and/or $G\alpha_o$ proteins. This inhibition exhibits three major biophysical characteristics: i) it slows down the activation kinetics of the inhibited channels, ii) shifts the activation voltage to a more depolarized potential and, iii) can be relieved by a strong conditioning depolarizing potential, resulting in the so-called prepulse facilitation [62,63]. These actions are mediated by the interaction of the G-protein $\beta\gamma$ dimer ($G\beta\gamma$) [64,65] with the alpha interacting domain (AID) located in the cytoplasmic loop (I-II loop) connecting the first two homologous repeats of the $Ca_V\alpha_1$ subunit (Fig. 2B) which also constitutes the high-affinity $Ca_V\beta$-binding site [66,67]. For this reason, and given that some functional effects of the $G\beta\gamma$ dimer are the opposites of those of $Ca_V\beta$ subunits, it had raised the question whether $G\beta\gamma$ and $Ca_V\beta$ compete with each other. However, recent studies suggest that the voltage dependence of $G\beta\gamma$ inhibition of HVA channels arises from the movement of IS6, and that $Ca_V\beta$ and a rigid IS6-AID linker play a pivotal role in translating this movement to $G\beta\gamma$ dissociation [68].

As mentioned earlier, the $Ca_V2\alpha_1$ subunit contains a synprint region that allows synaptic proteins to regulate channel activity [7,69,70], .i.e. binding of syntaxin 1 and SNAP-25 results in a hyperpolarizing shift in the half-inactivation potential, thus reducing channel availability [69,71,72]. However, a second action of syntaxin 1 on Ca_V channel regulation has been reported by Zamponi and his colleagues. Co-expression of this protein with N-type channels results in a tonic G protein-mediated channel inhibition that does not involve receptor activation [73]. This appears to be due to a syntaxin 1 mediated co-localization of the channel and the $G\beta\gamma$ dimer that ultimately culminates in tonic channel inhibition. Likewise, it is worth mentioning that the existence of a syntaxin-$Ca_V3.2$ (T-type) channel complex has been recently revealed in central neurons. Though this interaction

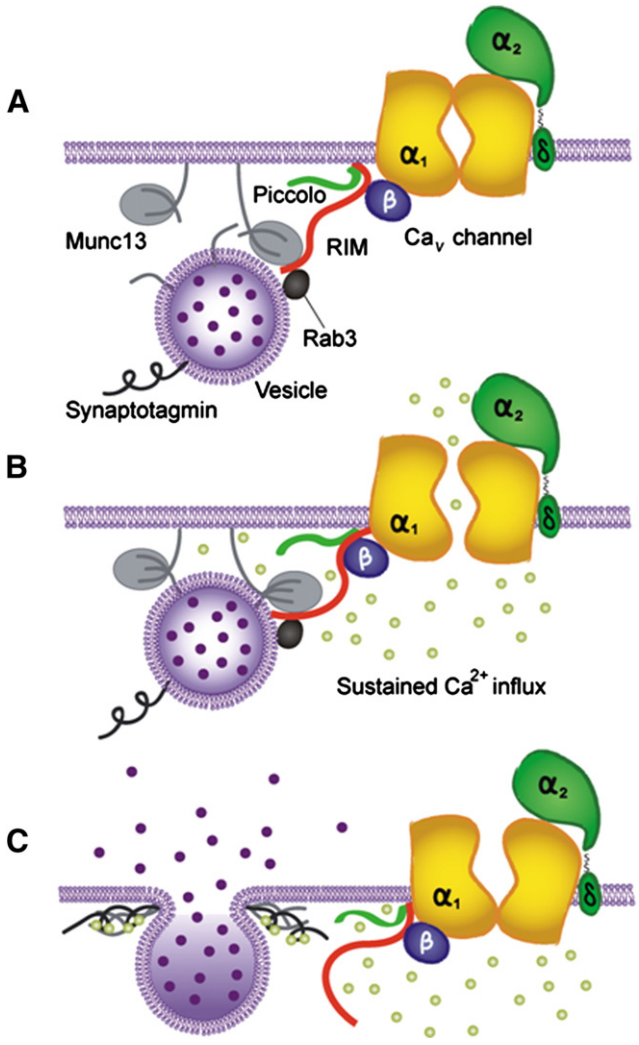

Fig. 3. Functional coupling between Ca_V channels and RIMs. A) RIMs anchor synaptic vesicles next to channels through its interaction with zone-specific proteins (Rab3, Munc13) and the $Ca_V\beta$ auxiliary subunit. B) After depolarization, RIMs regulate the time course of channel inactivation resulting in a sustained Ca^{2+} influx. D) These molecular interactions favor hormone and neurotransmitter release (C).

may involve different molecular determinants from those found in HVA channels, it modulates channel gating and appears essential for T-type channel-triggered low-threshold exocytosis [74].

Likewise, interesting actions of RIM1 on G-protein regulation of $Ca_V2.2$ channels heterologously expressed in HEK-293 have been recently reported [75]. As expected, RIM1 potently decreases the extent of $Ca_V2.2$ channel inactivation and the application of DAMGO, an agonist of the μ-opioid receptor, induced direct G-protein regulation. Interestingly, RIM1 did not alter the association of the Gβγ dimer onto the channel, but the kinetic and extent of recovery from G-protein inhibition were significantly affected in RIM1-expressing cells. These findings provided the first evidence for an efficient RIM1-dependent modulation of direct G-protein regulation of neuronal $Ca_V2.2$ channels, and emphasize the importance of the constitutive proteins from the presynaptic vesicle machinery, particularly RIMs, for both neurotransmitter secretion and fine-tuning of Ca_V channel activity [75].

In summary, neurotransmitter release is initiated by the activation of Ca_V channels close to docked vesicles. Although the mechanisms that enrich channels at release sites are largely unknown, recent studies suggest that RIM proteins may affect many aspects of neurotransmitter release [8–13, 76]. Combined, these studies support a model predicting a dual function for the RIM/Ca_V channel interaction in hormone and neurotransmitter release by coordinating the molecular constituents and Ca^{2+} signaling in the active zones (Fig. 3).

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